STIC-ILL

436,591 QP501.J7 mic. MPL

From: Pak, Yong
Sent: Monday, March 17, 2003 10:57 AM
To: STIC-ILL
Subject: 09/878,574

please find and copy the following for 09/878,574:

86081779 MEDLINE
DN 86081779 PubMed ID: 4076184
TI Purification and characterization of GDP-D-mannose 4,6-dehydratase from porcine thyroid.
AU Broschat K O; Chang S; Serif G
SO EUROPEAN JOURNAL OF BIOCHEMISTRY, (1985 Dec 2) 153 (2) 397-401. Journal code: 0107600. ISSN: 0014-2956.
CY GERMANY, WEST: Germany, Federal Republic of
DT Journal; Article; (JOURNAL ARTICLE)

Reitman ML, Trowbridge IS, Kornfeld S.
Mouse lymphoma cell lines resistant to pea lectin are defective in fucose metabolism.
J Biol Chem. 1980 Oct 25;255(20):9900-6.
PMID: 6159350 [PubMed - indexed for MEDLINE]

Two Chinese hamster ovary glycosylation mutants affected in the conversion of GDP-mannose to GDP-fucose.
AU Ripka J; Adamany A; Stanley P
NC 3PO CA13330 (NCI)
CA90173 (NCI)
R01 CA36434 (NCI)
SO ARCHIVES OF BIOCHEMISTRY AND BIOPHYSICS, (1986 Sep) 249 (2) 533-45. Journal code: 0372430. ISSN: 0003-9861.
CY United States

COMPLETED

Participation of an endogenous inhibitor of fucosyltransferase activities in the developmental regulation of intestinal fucosylation processes.
AU Ruggiero-Lopez D; Biol M C; Louisot P; Martin A
CS Department of General and Medical Biochemistry, INSERM-CNRS U. 189, France.
SO BIOCHEMICAL JOURNAL, (1991 Nov 1) 279 (Pt 3) 801-6. Journal code: 2984726R. ISSN: 0264-6021.

9980377

yong pak
Art Unit 1652

Tel: 703-308-9363
Fax: 703-746-3173
Office: 10A16
Mail: 10D01

THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 255, No. 20, Issue of October 25, pp. 9900-9906, 1980
Printed in U.S.A.

Mouse Lymphoma Cell Lines Resistant to Pea Lectin Are Defective in Fucose Metabolism*

(Received for publication, May 22, 1980, and in revised form, July 15, 1980)

Marc L. Reitman, Ian S. Trowbridge‡, and Stuart Kornfeld

From the Departments of Medicine and Biochemistry, Washington University School of Medicine, St. Louis, Missouri 63110 and the ‡Department of Cancer Biology, The Salk Institute for Biological Studies, San Diego, California 92138

Two mutants of the BW5147 mouse lymphoma cell line have been selected for their resistance to the toxic effects of pea lectin. These cell lines, termed PL^R1.3 and PHA^R1.8 PL^R7.2, have a decreased number of high affinity pea lectin-binding sites (Trowbridge, I. S., Hyman, R., Ferson, T., and Mazauskas, C. (1978) *Eur. J. Immunol.* 8, 716-723). Intact cell labeling experiments using [2-^{3}H]mannose indicated that PL^R1.3 cells have a block in the conversion of GDP-[^{3}H]mannose to GDP-[^{3}H]fucose whereas PHA^R1.8 PL^R7.2 cells appear to be blocked in the transfer of fucose from GDP-[^{3}H]fucose to glycoprotein acceptors. *In vitro* experiments with extracts of PL^R1.3 cells confirmed the failure to convert GDP-mannose to GDP-fucose and indicated that the defect is in GDP-mannose 4,6-dehydratase (EC 4.2.1.47), the first enzyme in the conversion of GDP-mannose to GDP-fucose. The block in the PL^R1.3 cells could be bypassed by growing the cells in the presence of fucose, demonstrating that an alternate pathway for the production of GDP-fucose presumably via fucose 1-phosphate is functional in this line. PL^R1.3 cells grown in 10 mM fucose showed normal high affinity pea lectin binding.

PHA^R1.8 PL^R7.2 cells synthesize GDP-fucose and have normal or increased levels of GDP-fucose:glycoprotein fucosyltransferase when assayed *in vitro*. The fucosyltransferases of this clone can utilize its own glycoproteins as fucose acceptors in *in vitro* assays. These findings indicate that this cell line fails to carry out the fucosyltransferase reaction *in vivo* despite the fact that it possesses the appropriate nucleotide sugar, glycoprotein acceptors, and fucosyltransferase.

The finding of decreased glycoprotein fucose in two independent isolates of pea lectin-resistant cell lines and the restoration of high affinity pea lectin binding to PL^R1.3 cells following fucose feeding strongly implicates fucose as a major determinant of pea lectin binding.

Cell lines selected for resistance to toxic concentrations of lectins have helped elucidate the pathways and biological roles of glycosylation and have helped define lectin-binding specificities (1). Previous work in our laboratories and in others has produced lectin-resistant cell lines with the following abnormalities: 1) defects in the biosynthesis of the lipid-linked oligosaccharide precursor of asparagine-linked glycoproteins (2-6); 2) a block in the processing of high mannose[1] species (7-9); and 3) modifications in the addition of outer sugars (*i.e.* *N*-acetylglucosamine, galactose, sialic acid, and fucose) to the processed core (8, 10-12).

In this paper we report the characterization of two pea lectin-resistant mutants of the BW5147 mouse lymphoma cell line. The mutants, PL^R1.3 and PHA^R1.8 PL^R7.2, were originally isolated and studied by Trowbridge *et al.* (10). The PHA^R1.8 PL^R7.2 line is a double mutant, produced by first isolating a BW5147 mutant resistant to kidney bean leucoagglutinin (PHA^R1.8)[2] and then selecting for resistance to pea lectin. The PHA^R1.8 cells, while having a decreased galactose and sialic acid content, have retained the parental cell line's sensitivity to pea lectin. Both pea lectin-resistant mutants show a decrease in pea lectin binding but have normal cell surface expression of three glycoprotein antigens (Thy-1, T-200, and gp69-71) (10).

We report here that the PL^R1.3 and PHA^R1.8 PL^R7.2 lines have less glycoprotein fucose than their respective parental cell lines. In the PL^R1.3 line this is due to a defect in the synthesis of the fucose donor, GDP-fucose. When this line is grown in the presence of fucose the biosynthetic defect is bypassed and the ability to bind pea lectin is restored. The defect in the PHA^R1.8 PL^R7.2 cells appears to be at the level of fucose transfer from GDP-fucose to glycoprotein.

EXPERIMENTAL PROCEDURES

Materials—Materials were obtained from the following sources: D-[2-^{3}H]mannose (18.4 Ci/mmol), L-[6-^{3}H]fucose (11.7 Ci/mmol), GDP-D-[1-^{3}H(N)]mannose (10.4 Ci/mmol), GDP-L-[U-^{14}C]fucose (0.140 Ci/mmol), and $Na^{125}I$, carrier free, New England Nuclear; α-minimal essential medium, Flow Laboratories, Rockville, MD; fetal bovine serum (heat inactivated), penicillin, and streptomycin, Grand Island Biological Co., Triton X-100 and 3a70 scintillation cocktail, Research Products International Corp., Elk Grove Village, IL; Bio-Gel P-6 (200 to 400 mesh) Bio-Rad Laboratories; Amberlite MB-3, Chemical Dynamics Corp., South Plainfield, NJ; and pea lectin, GDP-mannose, and sugars, Sigma. Pea lectin-Sepharose was made as previously described (13). Desialized fibrinogen glycopeptide was obtained from Dr. R. Kornfeld.[3] Dr. O. Gabriel, Georgetown Univer-

* This investigation was supported in part by Grants [illegible], CA 06369, CA 17213, and 5T05GM02016 from the United States Public Health Service and in part by National Institutes of Health Research Service Award GM 07200, Medical Scientist, from the National Institute of General Medical Sciences. A preliminary report of this work was presented at the American Society of Biological Chemists/Biophysical Society meeting, June 1980. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Unless otherwise noted, fucose is of the L configuration while all other sugars are of the D form.

[2] The abbreviations used are: PHA, kidney bean leucoagglutinin; GlcNAc, *N*-acetylglucosamine; PBS, phosphate-buffered saline (0.02 M $NaPO_4$, pH 7.4-0.14 M NaCl); PL, pea lectin.

[3] The original glycoprotein was obtained from Drs. J. Miletich and G. Broze of this institution. The structure of the oligosaccharide portion of the desialized Fib-1 glycopeptide is shown below (R.

sity Medical School, generously provided the 6-deoxy-L-talose standard.

Enzymes—Pronase was obtained from Calbiochem. Bovine epididymal α-L-fucosidase (EC 3.2.1.51) was purchased from Sigma. The fucosidase reaction mixtures contained glycopeptide and 0.012 unit of enzyme in 30 μl of 0.04 M sodium citrate, pH 4.5, and were incubated at 37°C for 48 h in a toluene atmosphere. Separation of free fucose from glycopeptide was accomplished by paper chromatography in Solvent C for 6 h.

Paper Chromatography—Descending paper chromatography was performed using either Whatman No. 1 or Whatman No. 3MM paper with the following solvent systems: Solvent A, 95% ethanol/1 M ammonium acetate, pH 7.5 (7:3); Solvent B, ethyl acetate/pyridine/water (8:2:1); Solvent C, ethyl acetate/pyridine/acetic acid/water (5:5:1:3); Solvent D, the upper phase from ethyl acetate/pyridine/water (360:100:115); and Solvent E, 1-butanol/pyridine/water (6:4:3). Authentic GDP-mannose was detected with ultraviolet illumination. Reducing sugar standards were visualized by the alkaline silver nitrate technique (14). Radiolabeled sugars were detected either with a TMC Vanguard Instrument Corp. model 885 scanner or by cutting the paper into 1-cm strips, adding 0.4 ml of water, 4 ml of 3a70, and counting in a scintillation spectrometer.

Protein Determinations—Protein concentrations were measured by the method of Lowry *et al.* (15) including 0.5% sodium dodecyl sulfate, with bovine serum albumin as the standard.

Cells—The selection of the mutants from the BW5147 (Thy-1$^+$) mouse lymphoma cell line has been described (10). Briefly, cells were mutagenized, cultured for 3 to 5 days without lectin (to allow expression of mutant phenotypes), cultured for 3 to 6 weeks in the presence of a toxic lectin concentration, and then cloned by limiting dilution. Cells were grown in suspension without agitation in α-minimal essential medium supplemented with either fetal calf or horse serum (10%), penicillin (50 units/ml), and streptomycin (50 μg/ml).

Labeling of Cells with Radioactive Sugars—In a typical experiment, 100 μCi of sugar were added to 20 ml of complete medium containing 5×10^4 cells per ml. After 2 or 3 days, as maximum cell density (1 to 2×10^6 cells per ml) was approached, the cells were harvested, washed twice with ice-cold phosphate-buffered saline, and frozen until extracted. Typically, about 3% of the added label remained cell associated at this point.

Extraction of Cells and Preparation of Glycopeptides—The pellet volume was brought to about 150 μl with carrier cells, and the cells were extracted successively with chloroform/methanol (2:1), water, and chloroform/methanol/water (10:10:3) by the method of Tabas *et al.* (16). The chloroform/methanol extract contains smaller lipid-linked oligosaccharides. The aqueous extract contains sugars, sugar phosphates, and nucleotide sugars, while the larger lipid-linked oligosaccharides are in the chloroform/methanol/water extracts. The pellet remaining after the extractions was suspended by sonication in 1 ml of 0.1 M Tris-Cl, pH 8.0, containing 2 mM $CaCl_2$. Pronase (2 mg) and toluene (a few drops) were added, and incubation at 37°C was begun. Additional pronase (2 mg) was added daily for 3 days, and then the reaction mixture was heated for 5 min in a boiling water bath. Precipitated material was removed by centrifugation, and the supernatant containing the glycopeptides was desalted on a column (0.9 × 28 cm) of Sephadex G25-80.

Assay of Aqueous Extracts for Nucleotide Sugars—The pooled aqueous extracts were concentrated under reduced pressure, spotted on Whatman No. 3MM, and chromatographed in Solvent A for 43 h. This system separates nucleotide sugars from sugar phosphates, free sugars, and glycoproteins, but does not separate GDP-mannose from GDP-fucose (17). The nucleotide sugar region was eluted with water, and an aliquot was brought to pH 2 with HCl and hydrolyzed for 10 min at 100°C. The hydrolysate was neutralized with NaOH and spotted on Whatman No. 1 paper which was developed in Solvent B for 21 h. Unhydrolyzed nucleotide sugar served as a control. The radioactivity in the mannose and fucose areas was counted and used as the measure of the GDP-mannose and GDP-fucose present in the initial sample.

Sugar Analysis of Labeled Glycopeptides—Identification of the labeled sugars present in a given glycopeptide was performed as follows. The glycopeptide was hydrolyzed in 2 N H_2SO_4 for 4 h at 100°C in a final volume of 150 μl. After dilution with water, the sample was desalted on a column (0.5 × 8.5 cm) of Amberlite MB-3 and concentrated. An aliquot was spotted on Whatman No. 1 paper, chromatographed in Solvent B for 20 h, and radioactivity was determined as described above.

Fucose Content of Particulate Fractions—Parent (3×10^9) and PL^{R}1.3 (6×10^9) cells were each suspended in 1 volume of PBS, sonicated briefly, and sedimented at 130,000 × *g* for 45 min. The crude membrane pellets were washed once and resuspended in 8 ml of 0.2 N H_2SO_4 containing 10^5 cpm of [^{3}H]fucose as tracer. The suspension was hydrolyzed for 11 h at 100°C, and particulate material was removed by centrifugation. The pellet was washed twice with 10 ml of H_2O, and the three supernatant fractions were combined, desalted on a column (0.9 × 15 cm) of Amberlite MB-3, and lyophilized. The samples were then streaked on Whatman No. 3MM and chromatographed for 21 h in Solvent B. The fucose-containing region was eluted and rechromatographed in Solvent B. The areas corresponding to a fucose standard was eluted, and the fucose content was determined by gas-liquid chromatography as previously described (18). We recovered 45% of the [^{3}H]fucose tracer. A blank sample was carried through all the steps subsequent to the desalting. It did not contain any fucose or other interfering compounds.

In Vitro Conversion of GDP-D-Mannose to GDP-L-Fucose—This procedure is adapted from those of Foster and Ginsburg (19) and Kornfeld and Ginsburg (20). Cell extracts were prepared daily according to the following procedure; all steps were carried out at 4°C. Cells were washed with PBS, suspended in 10 to 20 volumes of 0.05 M Tris-Cl, pH 8.0, and disrupted by sonication (two 15-s pulses of a Bronwil Biosonik IV, set at 60 using a microprobe). After centrifugation (130,000 × *g*, 60 min), 100 to 400 μg of cytosolic protein were added to assay mixtures (1.0 ml final volume) containing 0.2 μmol of NADP$^+$, 4.0 μmol of glucose 6-phosphate, 0.2 μmol of NAD$^+$, 5.0 μmol of ATP, 10 μmol of nicotinamide, 3.0 nmol of GDP-[1-^{3}H]mannose (8000 cpm), and 50 mM Tris-Cl, pH 8.0. The glucose 6-phosphate and NADP$^+$ are used by endogenous glucose 6-phosphate dehydrogenase to produce NADPH. The ATP and nicotinamide serve as carrier. After a 60-min incubation at 37°C, 20 μl of 2 M HCl were added, and the reaction mixtures were heated at 100°C for 10 min to hydrolyze the nucleotide sugars. The samples were desalted on Amberlite MB-3, subjected to paper chromatography in Solvent B, and the radioactivity in the mannose and fucose regions determined.

To prove that the fucose detected by this assay is derived from GDP-fucose, a similar reaction was terminated by boiling in the absence of acid. The nucleotide sugars were absorbed onto 60 mg of acid-washed Norit A, washed twice with 5 ml of 1 mM HCl, and then eluted three times with 5 ml of 50% ethanol containing 0.1% NH_4OH. The eluted fractions were concentrated under reduced pressure, spotted on Whatman No. 1 paper, and chromatographed in Solvent A. The nucleotide sugar region was eluted with water, hydrolyzed (0.01 M HCl, 10 min, 100°C), neutralized, and chromatographed in Solvent B to determine the radioactivity in mannose and fucose. This was compared to values obtained in the standard assay.

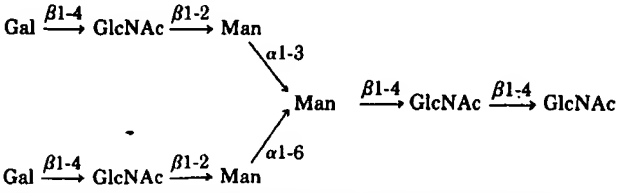
^{125}I-Pea Lectin-binding Studies—Pea lectin was iodinated by the method of Greenwood *et al.* (21) using a 15-s exposure to chloramine T. The iodinated lectin was immediately adsorbed to a column (0.5 × 4 cm) of Sephadex G75-120, washed with 10 mM Tris-Cl, pH 8.0, 0.15 M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% (w/v) NaN_3, and eluted with 0.10 M α-methyl glucoside in the same buffer. Bovine serum albumin was added to a final concentration of 4 mg/ml, and the solution was dialyzed against the wash buffer. Binding assays were performed as previously described (10) except that the reactions were terminated by centrifuging the cells through an oil layer, leaving behind the aqueous phase containing the free lectin (22).

RESULTS

In Vivo Labeling with [2-^{3}H]Mannose—Since it seemed likely that the pea lectin-resistant cell lines had altered cell surface oligosaccharides, our initial experiment was to grow the parent and mutant lines in the presence of [2-^{3}H]mannose in order to label the mannose and fucose residues of these

---

Kornfeld, unpublished data).



oligosaccharides. After 3 days the cells were harvested, and glycopeptides were prepared by pronase digestion. An aliquot of the glycopeptide fraction was subjected to acid hydrolysis, and the released sugars were separated by paper chromatography. As shown in Table I, about 90% of the radioactivity was recovered from the parent BW5147 and the $PHA^R1.8$ lines as [^{3}H]mannose and the rest as [^{3}H]fucose. In contrast, the glycopeptides of the two pea lectin-resistant lines ($PL^R1.3$ and $PHA^R1.8$ $PL^R7.2$) were strikingly deficient in [^{3}H]fucose.

The decreased fucose content of the glycoproteins from the pea lectin-resistant lines could result from any of a number of mechanisms. These include defects in: 1) the synthesis of GDP-fucose from GDP-mannose, 2) the transport of GDP-fucose into the Golgi apparatus, and 3) the transfer of fucose to glycoprotein acceptor(s). To examine the first possibility, we analyzed the aqueous fractions of the [2-^{3}H]mannose-labeled cells for the presence of labeled GDP-mannose and GDP-fucose. Extracts from all the cell lines contained GDP-[^{3}H]mannose. GDP-[^{3}H]fucose was readily detected in the extracts of the parent BW5147 and the $PHA^R1.8$ $PL^R7.2$ cells. However, in two separate experiments, we were unable to detect any radioactive GDP-fucose in the $PL^R1.3$ extracts (Table II).

In Vitro Conversion of GDP-Mannose to GDP-Fucose—The preceding experiments suggest that the defect in the $PL^R1.3$ cells is an inability to convert GDP-mannose to GDP-fucose. Therefore, an *in vitro* assay was established to examine this conversion and to allow manipulation of the reaction conditions. Cell extracts were incubated with GDP-[^{3}H]mannose, NAD^+, and a source of NADPH, and the extent of GDP-[^{3}H]fucose formation was determined by measuring the [^{3}H]fucose present in mild acid hydrolysates of the incubation mixtures. In the standard assay, [^{3}H]fucose formation was proportional to the amount of protein added, at protein concentrations above 100 μg/ml (Fig. 1). After a 5- to 10-min lag, [^{3}H]fucose production was linear with time. While NAD^+ was included in the reaction mixtures, it was not required for full enzymatic activity.

In order to prove that the [^{3}H]fucose detected by this assay is derived from GDP-[^{3}H]fucose, we scaled up the standard incubation mixture, terminated the reaction by boiling in the absence of acid, and isolated the nucleotide sugars (see under "Experimental Procedures"). When these nucleotide sugars were hydrolyzed and the released monosaccharides isolated, [^{3}H]fucose was found to make up 32% of the recovered radioactivity. In comparison, when the assay was performed using the standard procedure, [^{3}H]fucose comprised 23% of the recovered label. [^{3}H]Mannose accounted for the remainder of the radioactivity in both cases. This agreement demonstrates that the assay reflects the conversion of GDP-mannose to GDP-fucose.

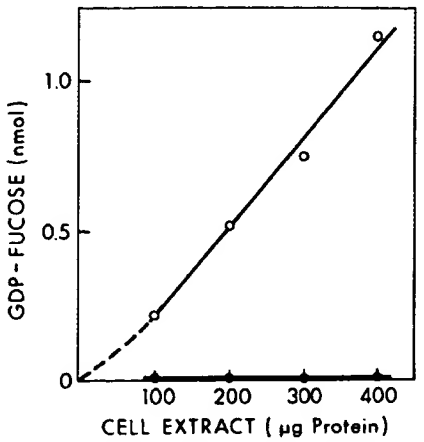


FIG. 1. **Ability of cell extracts to convert GDP-[^{3}H]mannose to GDP-[^{3}H]fucose.** Soluble cell extracts were prepared and assayed as described under "Experimental Procedures," except that the incubation time was 70 min. The soluble cell extracts contained 30 to 45% of total cell protein and greater than 90% of the GDP-mannose to GDP-fucose converting activity. O——O, parent BW5147 extract; ●——●, $PL^R1.3$ extract.

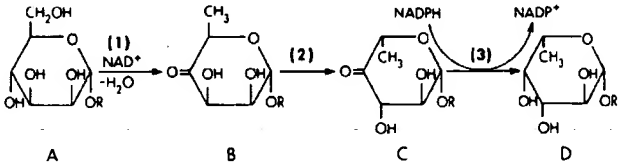


FIG. 2. **Proposed pathway for the biosynthesis of GDP-fucose from GDP-mannose.** From Ginsburg (23); *R* = GDP. Compounds are: *A*, GDP-α-D-mannose; *B*, GDP-6-deoxy-α-D-lyxo-4-hexulose (GDP-4-keto-6-deoxy-α-D-mannose); *C*, GDP-6-deoxy-β-L-xylo-4-hexulose (GDP-4-keto-6-deoxy-β-L-galactose); and *D*, GDP-6-deoxy-β-L-galactose (GDP-β-L-fucose). Enzymatic activities are: (*1*) 4,6-dehydratase (oxidoreductase); (*2*) 3,5-epimerase; and (*3*) 4-reductase.

The data in Fig. 1 show that $PL^R1.3$ cell extracts lack the ability to convert GDP-mannose to GDP-fucose. Unlike the $PL^R1.3$ line, $PHA^R1.8$ $PL^R7.2$ cell extracts demonstrated the same ability to convert GDP-mannose to GDP-fucose as did parental cells (data not shown).

The conversion of GDP-mannose to GDP-fucose requires at least three different enzymatic activities, as shown in Fig. 2 (23-25). In a bacterial system, incubation without NADPH will cause intermediate compounds (*B* and possibly *C* in Fig. 2) to accumulate. Then, upon addition of NADPH, GDP-fucose rapidly appears without the usual time lag (23). We, therefore, performed a series of experiments to determine if the conversion of GDP-mannose to GDP-fucose in mouse cells follows the same pathway as in *Aerobacter aerogenes* and then took advantage of these results to localize more precisely the defect in the $PL^R1.3$ line. First, we examined the ability of the parent and $PL^R1.3$ extracts to product interme-

TABLE I

Radioactive sugars present in glycopeptides after in vivo labeling with [2-^{3}H]mannose

The labeling of cells, preparation of the glycopeptides, and the sugar analysis were performed as described under "Experimental Procedures." Results are expressed as the percentage of total radioactivity that is present in the indicated sugar. Each assay was performed using at least 3000 cpm of glycopeptide.

Cell line	Percentage of radioactivity present in	
	Fucose	Mannose
Parent BW5147	10.3	89.7
$PL^R1.3$	<0.5	>99.5
$PHA^R1.8$	10.2	89.8
$PHA^R1.8$ $PL^R7.2$	0.96	99.0

TABLE II

Radiolabeled nucleotide sugars present in cell extracts after in vivo labeling with [2-^{3}H]mannose

The labeling of cells, isolation of nucleotide sugars, mild acid hydrolysis, and identification of the released monosaccharides were performed as described under "Experimental Procedures."

Cell line	Radioactivity in	
	GDP-fucose	GDP-mannose
	cpm	
Parent BW5147	3460	1519
$PL^R1.3$	<25	982
$PHA^R1.8$ $PL^R7.2$	1566	3577

diates *B* and *C* from GDP-mannose. Cell extracts were incubated in reaction mixtures which lacked a source of NADPH in order to accumulate the 4-keto intermediates. At the end of the incubation time $NaBH_4$ was added to reduce any 4-keto intermediates. The nucleotide sugars were then hydrolyzed, and the released sugars separated by paper chromatography. When compound *B* is reduced and hydrolyzed, 6-deoxy-D-talose (D-talomethylose) and 6-deoxy-D-mannose (D-rhamnose) are produced, while intermediate *C* will produce 6-deoxy-L-galactose (L-fucose) and 6-deoxy-L-glucose (23). If the 3- and 5-epimerase activities are separate enzymes, additional 6-deoxyhexoses might be detected.

The results of the trapping experiments are presented in Table III. The incubation with parental extracts yielded radioactivity that migrated with mannose, 6-deoxytalose, and rhamnose. Additional proof that the radioactivity was due to the indicated sugars was obtained by rechromatographing the individual peaks in Solvents B, D, and E. Authentic D-mannose, L-fucose, L-rhamnose, 6-deoxy-L-talose, and 6-deoxy-D-glucose were used as standards. The material labeled 6-deoxytalose in Table III cochromatographed with the standard in all three solvents. The radioactivity that chromatographed in the rhamnose area in Solvent D was predominantly rhamnose but may have contained small amounts (<10%) of 6-deoxyglucose and fucose. These data are consistent with Ginsburg's proposed pathway for the conversion of GDP-mannose to GDP-fucose (23). The data also suggest that when 4-reductase activity is inhibited, intermediate *B* accumulates to a much greater extent than *C*.

When the reductive trapping experiment was performed using $PL^R1.3$ extracts, no 6-deoxyhexoses were detected. This finding suggests that the defect in the $PL^R1.3$ line is in the step catalyzed by GDP-mannose 4,6-dehydratase (EC 4.2.1.47, also known as GDP-mannose, 4,6-hydrolyase and GDP-mannose 4,6-oxidoreductase). The data rule out a deficient 3,5-epimerase as the sole enzyme defect in $PL^R1.3$ cells, but it does not exclude the possibility that the cell lacks both this enzyme and the dehydratase.

To determine whether the epimerase and reductase activities are present in the $PL^R1.3$ line, we performed the experiment shown in Fig. 3. Parent or $PL^R1.3$ extracts were preincubated without NADPH to allow the formation of intermediates, boiled to inactivate the extracts, and then incubated a second time with fresh extracts as detailed in the figure legend. *Panel A* contains control samples, demonstrating that this preparation of parental extract produces 0.40 nmol of GDP-fucose in 1 h, after a time lag, and that the $PL^R1.3$ extract cannot synthesize GDP-fucose. The data in *Panel B* show that preincubation with $PL^R1.3$ extracts without NADPH does not affect GDP-fucose formation during a second incubation with parental extract. In contrast, as illustrated in *Panel C*, preincubation with parental extract produces intermediates that $PL^R1.3$ extracts can convert to GDP-fucose, and this conversion occurs without the time lag. Because *B* is probably the predominant intermediate (above), the most likely interpretation of these results is that $PL^R1.3$ extracts can perform both epimerase and reductase functions. However, we cannot exclude the possibility that one (but not both) of these activities was not inactivated by boiling and thus was present from the preincubations.

When parental cell extracts were mixed with $PL^R1.3$ extracts, the conversion of GDP-mannose to GDP-fucose was identical with that observed in control incubations with pa-

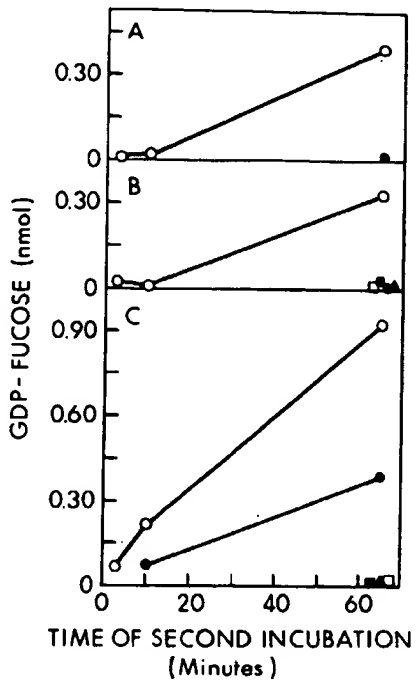


FIG. 3. **Conversion of nucleotide sugar intermediates to GDP-fucose by cell extracts.** GDP-[^{3}H]mannose was preincubated with 250 μg of soluble protein in 800-μl final volume containing substrates and carrier as described under "Experimental Procedures," except that $NADP^+$ and glucose 6-phosphate were omitted. After 65 min of preincubation, the reaction mixtures were placed in a boiling water bath for 105 s to inactivate the extracts. The preincubations were performed as indicated. *Panel B*, preincubation with $PL^R1.3$ cell extract; *Panel C*, preincubation with parental BW5147 extract; *Panel A* represents a control in which no preincubation was performed. The second incubations were initiated by the addition of 250 μg of protein, 0.2 μmol, NAD^+, 10 μmol of nicotinamide, and 5.0 μmol of ATP in 50 mM Tris-Cl, pH 8.0 (with or without 0.2 μmol of $NADP^+$ and 4.0 μmol of glucose 6-phosphate, as indicated) in a volume of 290 μl. In the incubations without fresh cell extract, 0.3 μmol of NADPH was added instead of $NADP^+$ and glucose 6-phosphate. The second incubations with either fresh parental (○, □) or $PL^R1.3$ (●, ■) extract and either with a source of NADPH (○, ●) or without one (□, ■) were performed for the indicated times. ▲, reactions with NADPH but without cell extract.

TABLE III

Radioactive compounds found after incubating GDP-[^{3}H]mannose with cell extracts in the absence of NADPH, followed by reduction and mild acid hydrolysis

GDP-[^{3}H]mannose (3.68×10^5 cpm; 0.035 nmol) was incubated with 400 μg of supernatant protein as described under "Experimental Procedures" except that exogenous $NADP^+$ and glucose 6-phosphate were omitted. After 60 min at 37°C, the reaction mixtures were heated for 105 s in a boiling water bath, and insoluble protein was removed by centrifugation at 12,000 × *g* for 10 min. $NaBH_4$ (1 mg) was then added to the supernatant fluid. After 3 h at room temperature, 60 μl of 2 N HCl was added, and the reactions were incubated at 100°C for 10 min to hydrolyze the nucleotide sugars. The samples were desalted on Amberlite MB-3 and chromatographed in Solvent D for 11 h. The paper was cut into 1-cm strips and counted for radioactivity. In addition to radioactivity in the mannose, rhamnose, and 6-deoxytalose regions, a small peak was detected with a mobility between that of rhamnose and 6-deoxytalose. This accounted for 0.5% of the radioactivity in the $PL^R1.3$ and control chromatograms and was not detectable in the chromatogram of parent cells due to tailing from the 6-deoxytalose and rhamnose peaks. It appears to represent a trace contaminant in the starting GDP-[^{3}H]mannose.

Cell extract	Mannose	Rhamnose	6-Deoxytalose
		cpm	
None	128,120	<120	<120
Parent BW5147	61,295	2,459	4,484
$PL^R1.3$	64,564	<120	<120

rental extract alone (data not shown). This experiment demonstrated that $PL^R1.3$ cells do not contain a soluble inhibitor of the 4,6-dehydratase and that the parental cell extracts do not provide compounds (*e.g.* NADPH) that enable the GDP-mannose to GDP-fucose pathway to function in $PL^R1.3$ extracts. The mixing data also suggest that the defective reaction in $PL^R1.3$ is the rate-determining step of the conversion pathway.

Assaying the "Scavenge" Pathway of Fucose Metabolism in $PL^R1.3$—While the predominant pathway for GDP-fucose formation involves the conversion of GDP-mannose to GDP-fucose, a "scavenge" pathway also exists whereby GDP-fucose is formed from free fucose via fucose 1-phosphate (26). We assayed for the presence of this pathway in parent and $PL^R1.3$ cells by growing the cells in media containing [^{3}H]fucose. Both cell lines incorporated [^{3}H]fucose into GDP-[^{3}H]fucose, demonstrating that the scavenge pathway is functional in these cells (data not shown). We next subjected the glycoproteins from these [^{3}H]fucose-labeled cells to mild base treatment to release *O*-glycosidically linked oligosaccharides followed by pronase digestion and Bio-Gel P-6 gel filtration chromatography (Fig. 4). The elution profiles of the parental and $PL^R1.3$ material were very similar, with most of the radioactivity eluting as glycopeptide species at or near the void volume. Acid hydrolysis of the glycopeptides followed by paper chromatography confirmed that all the label was in fucose.

To determine the magnitude of the scavenge pathway in $PL^R1.3$ cells under normal culture conditions, we assayed unlabeled parent and $PL^R1.3$ cells for the amount of fucose present in total cellular membrane material. The washed cells

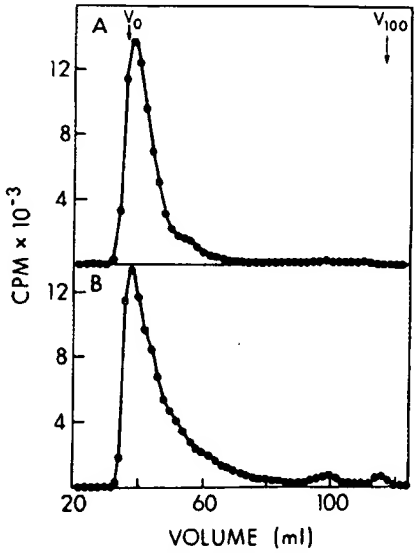


FIG. 4. **Parent and $PL^R1.3$ cells incorporate radioactivity into glycoprotein upon incubation with [^{3}H]fucose.** Cells (8.3×10^7 parent and 5.4×10^7 $PL^R1.3$ at harvesting) were labeled (50 μCi of [^{3}H]fucose/35 ml, 83 h) harvested, and extracted, as described under "Experimental Procedures." The resulting residual glycoprotein was subjected to mild base treatment (0.1 M NaOH, 0.4 M $NaBH_4$, under N_2 in the dark at room temperature for 68 h) followed by neutralization (0.1 M acetic acid) and removal of the borate (addition of methanol followed by evaporation to dryness under reduced pressure, repeated three times). After pronase digestion, the glycopeptides were chromatographed on a column (100 × 1.5 cm) of Bio-Gel P-6 using 0.1 M NH_4HCO_3 as the column buffer. Each fraction was 2 ml, 10% of which was used for determination of radioactivity. The void volume (V_0) was measured with bovine serum albumin, while glucose served as the included volume (V_{100}) marker. The elution profile of parent BW5147 glycopeptides is in *Panel A* and that of $PL^R1.3$ glycopeptides is presented in *Panel B*.

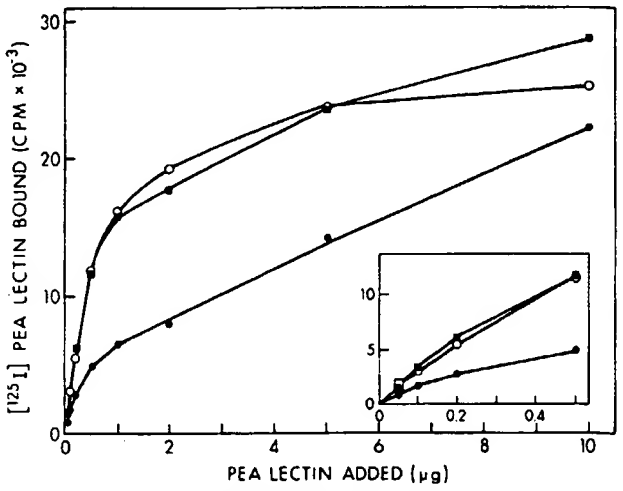


FIG. 5. **Specific binding of ^{125}I-labeled pea lectin to parent BW5147, $PL^R1.3$, and $PL^R1.3$ cells grown in 10 mM fucose.** The $PL^R1.3$ cells were grown in complete medium containing 10 mM fucose for 3 days. Binding reactions (100 μl) contained 5×10^5 cells, ^{125}I-labeled pea lectin (6.7×10^4 cpm/μg), 0.5 mg of bovine serum albumin, and 1 μmol of NaN_3 in PBS. Incubations were at 4°C for 3 h. Separation of cells from free lectin was performed as described (22). Specific binding is defined as binding that is inhibitable by 0.1 M α-methyl mannoside. Specific binding accounted for greater than 90% of the total pea lectin bound below 1 μg added and for >80% of the lectin bound above this concentration. ○, parent BW5147; ●, $PL^R1.3$; ■, $PL^R1.3$ grown for 3 days with 10 mM exogenous fucose. The *inset* shows lectin binding at the low lectin concentrations.

were disrupted by sonication, and the particulate fractions were sedimented. The pellets were then hydrolyzed and the free fucose purified by paper chromatography and assayed by gas-liquid chromatography (see under "Experimental Procedures" for details). Wild type cells contained 1.52 nmol of fucose/mg of particulate protein compared to 0.33 nmol of fucose/mg of particulate protein in $PL^R1.3$ cells.

Restoration of Pea Lectin Binding to $PL^R1.3$ Cells by Fucose Feeding—We next cultured the cells in medium containing substrate amounts of fucose. These experiments had two purposes, to further demonstrate the functionality of the scavenge pathway and to examine the role of fucose in pea lectin binding. Cells were grown in the presence of 1 mM and 10 mM fucose, since higher concentrations of this sugar (20 to 80 mM) had been shown to cause changes in cell morphology and inhibition of cell growth (27-29). Control experiments demonstrated that 1 mM and 10 mM fucose did not inhibit the growth of parent cells. Fig. 5 shows the specific binding of ^{125}I-labeled pea lectin to parental and $PL^R1.3$ cells and to $PL^R1.3$ cells grown in 10 mM fucose. It is evident that fucose feeding restores the ability of $PL^R1.3$ cells to bind pea lectin. Scatchard plots of the data demonstrate that $PL^R1.3$ cells have approximately one-third the number of high affinity pea lectin-binding sites as either fucose-fed $PL^R1.3$ cells or parental cells.

Two other characteristics that distinguish $PL^R1.3$ cells from the parent BW5147 line are the mutant's prolonged doubling time (28 h *versus* 15 h for parent cells) and their more spherical morphology. Neither of these traits was affected by growing the $PL^R1.3$ cells in 1 or 10 mM fucose for 6 days.

In Vitro Fucosyltransferase Assays—Data presented above indicate that the $PHA^R1.8$ $PL^R7.2$ line's defect may be at the site of fucose transfer from GDP-fucose to glycoprotein. In order to test this hypothesis, we assayed the ability of a detergent extract from each cell line to transfer fucose from GDP-fucose to exogenous fibrinogen glycopeptide and to endogenous acceptors. Table IV contains representative results from one of four experiments. The extract of each mutant line

TABLE IV

GDP-fucose: glycoprotein fucosyltransferase activity

Washed cells (1×10^8) in 8 volumes of 20 mM Tris-maleate, pH 7.4, containing 1% (v/v) Triton X-100 were disrupted by sonication and centrifuged at 12,000 × *g* for 10 min. Incubation mixtures (30) of 50 μl contained 500 μg of the supernatant protein, 40 mM $MgCl_2$, 1% (v/v) Triton X-100, 5 mM ATP, 14 μM GDP-[^{14}C]fucose (10^5 cpm), 20 mM Tris-maleate, pH 7.4, and 2 mM fibrinogen glycopeptide where indicated. After incubating for 5.5 h at 37°C, the entire reaction mixtures were spotted on Whatman No. 3MM paper and developed in Solvent C for 10 h. In this system, the glycopeptide stays at the origin while the GDP-fucose and free fucose migrate with R_f values of 0.18 and 0.61, respectively. All assays were performed in duplicate, and the maximum variation was 11%.

Source of cell extract	Fib-1 acceptor glycopeptide[a]	Radioactivity at origin
		cpm
None	+	666
Parent BW5147	−	600
	+	15,466
$PL^R1.3$	−	986
	+	40,488
$PHA^R1.8\ PL^R7.2$	−	2,467
	+	38,652

[a] Structure shown in footnote 3.

transferred fucose to the exogenous fibrinogen glycopeptide acceptor as well or better than parent cell extracts did. $PHA^R1.8\ PL^R7.2$ extracts transferred as much or more radioactivity to endogenous acceptors than did either parent or $PL^R1.3$. Thus, both $PHA^R1.8\ PL^R7.2$ and $PL^R1.3$ exhibit fucosyltransferase activity in an *in vitro* assay. A simple lack of the fucosyltransferase enzyme can be ruled out as the enzymatic defect in $PHA^R1.8\ PL^R7.2$.

α-L-Fucosidase digestion of the fucosylated fibrinogen glycopeptide product released 78 to 95% of the radioactivity as free fucose, demonstrating that the transferred fucose is in α-linkage. Further characterization of the product was obtained using pea lectin-Sepharose chromatography. The fibrinogen glycopeptide substrate did not adhere to a pea lectin-Sepharose column, while 78 to 94% of the ^{14}C-fucosylated glycopeptide product bound to the column and could be eluted with 10 mM α-methylglucoside (data not shown). These results indicate that the fucose residue(s) transferred *in vitro* is necessary for pea lectin binding.

DISCUSSION

The pea lectin-resistant lines analyzed in this study appear to have novel defects in glycoprotein biosynthesis. $PL^R1.3$ cells have an absent or greatly diminished ability to convert GDP-mannose to GDP-fucose while the intact $PHA^R1.8\ PL^R7.2$ cells are unable to transfer fucose from GDP-fucose to glycoprotein acceptors. Although the mechanism of the conversion of GDP-mannose to GDP-fucose has been examined only in bacteria (23), this pathway has been explicitly demonstrated in rabbit tissues (19) and is presumed to be present in the many tissues and cell lines that incorporate radioactivity into fucose after incubation with labeled mannose. The analogous bacterial metabolism of dTDP-D-glucose to dTDP-L-rhamnose and dTDP-6-deoxy-D-talose has been extensively studied and shown to involve at least three physically separable enzymes, a 4,6-oxidoreductase (4,6-dehydratase), a 3,5-epimerase, and a 4-reductase (24, 25, 31). Our results are consistent with the hypothesis that the eukaryotic mouse lymphoma cells use the same pathway as do bacteria and that at least three enzymatic activities are involved in the metabolism of GDP-mannose to GDP-fucose. We have shown that the defect in $PL^R1.3$ cells is in GDP-mannose 4,6-dehydratase, the enzyme that converts GDP-mannose to GDP-4-keto-6-deoxymannose.

The defect in the $PHA^R1.8\ PL^R7.2$ line is more difficult to discern at the enzymatic level. The intact cells appear to be unable to transfer fucose from GDP-fucose to glycoprotein acceptors in spite of the fact that they can synthesize GDP-fucose and appear to have normal or even increased levels of fucosyltransferase activity as well as the appropriate endogenous acceptors. There are at least four possible explanations consistent with these findings. 1) GDP-fucose may not be transported into the Golgi lumen, resulting in a lack of substrate for the fucosyltransferases. 2) The fucosyltransferases may not be properly located within the cell. This would not have been detected in the *in vitro* assays since detergent was present. 3) A glycosyltransferase may be induced which forms a product that is not an acceptor for the fucosyltransferase. Beyer *et al.* have demonstrated that the synthesis of some oligosaccharides requires a specific order of sugar addition and also that the actions of certain glycosyltransferases are mutually exclusive (32). However, the finding of increased transfer of fucose to endogenous acceptors in the $PHA^R1.8\ PL^R7.2$ line is against this explanation. 4) A hyperactive fucosidase could be present. This explanation is improbable because one would expect such an enzyme to be active during the *in vitro* fucosyltransferase assays, resulting in an apparent decrease in fucose transfer to glycopeptide. This was not observed.

A number of other lectin-resistant cell lines fail to carry out specific glycosyltransferase reactions *in vivo*, although they possess the appropriate nucleotide sugars, glycoprotein and glycolipid acceptors, and glycosyltransferases. Clone 13, a wheat germ agglutinin-resistant Chinese hamster ovary cell line, is deficient in galactose, but when assayed *in vitro* it exhibits normal UDP-galactose:glycoprotein galactosyltransferase and UDP-galactose:glucosyl ceramide galactosyltransferase activities (11). Another wheat germ agglutinin-resistant cell line which is deficient in sialic acid contains CMP-sialic acid and *in vitro* sialyltransferase activity (11). Finally, $PHA^R1.8$, the immediate parent to $PHA^R1.8\ PL^R7.2$, is similar to clone 13. It exhibits galactosyltransferase activity but has reduced glycoprotein galactose, even when grown in galactose to circumvent any possible UDP-glucose:UDP-galactose epimerase deficiency (10).

The finding of diminished glycoprotein fucose content in two separate pea lectin-resistant lines with different enzymatic defects points to a critical role for fucose in the pea lectin cell surface receptor. The restoration of high affinity pea lectin binding when $PL^R1.3$ cells are grown in fucose-containing media supports this postulate. In experiments to be published elsewhere, evidence has been obtained that the fucose residue linked to the inner core *N*-acetylglucosamine of biantennary complex-type glycopeptides is essential for high affinity binding to pea lectin.[4] A previous study of the carbohydrate binding specificity of pea lectin did not examine the role of fucose in the interaction (33).

The fucose residues of oligosaccharides are known to participate in a number of biologic reactions. They serve as blood group determinants (34), as the receptor for macrophage migration inhibitory factor (35–37), and as the determinant recognized by the hepatic fucose (38) and macrophage mannose/fucose receptors (39). Fucose has also been implicated in sperm-egg binding (40), as one of the determinants of the T locus antigens of the mouse (41), and, possibly, in lymphocyte homing (42). A B16 melanoma cell line with a decreased

[4] K. Kornfeld, M. Reitman, and R. Kornfeld, manuscript in preparation.

tendency to metastasize contains glycoproteins that have a decreased sialic acid content and an increased fucose content (12). The finding that pea lectin may be used to select cell lines with defects in fucose metabolism may aid future investigations of the biologic roles of this sugar.

Acknowledgments—We are very grateful to Dr. Jacques Baenziger for help with the gas-liquid chromatography and to Ms. Karen Fujimoto for assistance with the cell culturing.

REFERENCES

1. Briles, E. B., and Kornfeld, S. (1978) *Trends Biochem. Sci.* **3**, 223–227
2. Trowbridge, I. S., and Hyman, R. (1979) *Cell* **17**, 503–508
3. Chapman, A., Trowbridge, I. S., Hyman, R., and Kornfeld, S. (1979) *Cell* **17**, 509–515
4. Robertson, M. A., Etchison, J. R., Robertson, J. S., Summers, D. F., and Stanley, P. (1978) *Cell* **13**, 515–526
5. Krag, S. S. (1979) *J. Biol. Chem.* **254**, 9167–9177
6. Briles, E. B., Schlesinger, S., and Kornfeld, S. (1978) *J. Cell Biol.* **79**, 405A
7. Gottlieb, C., Baenziger, J., and Kornfeld, S. (1975) *J. Biol. Chem.* **250**, 3303–3309
8. Stanley, P., and Siminovitch, L. (1977) *Somat. Cell Genet.* **3**, 391–405
9. Meager, A., Ungkitchanukit, A., Nairn, P., and Hughes, R. C. (1975) *Nature* **257**, 137–139
10. Trowbridge, I. S., Hyman, R., Ferson, T., and Mazauskas, C. (1978) *Eur. J. Immunol.* **8**, 716–723
11. Briles, E. B., Li, E., and Kornfeld, S. (1977) *J. Biol. Chem.* **252**, 1107–1116
12. Finne, J., Tao, T.-W., and Burger, M. M. (1979) *Fifth International Symposium on Glycoconjugates* (Schauer, R., ed) pp. 609–610, Georg Thieme, Stuttgart
13. Trowbridge, I. S., Nilsen-Hamilton, M., Hamilton, R. T., and Bevan, M. J. (1977) *Biochem. J.* **163**, 211–217
14. Trevelyan, W. E., Proctor, D. P., and Harrison, J. S. (1950) *Nature* **166**, 444–445
15. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
16. Tabas, I., Schlesinger, S., and Kornfeld, S. (1978) *J. Biol. Chem.* **253**, 716–722
17. Yurchencho, P. D., Ceccarini, C., and Atkinson, P. H. (1978) *Methods Enzymol.* **50**, 175–204
18. Baenziger, J., Kornfeld, S., and Kochwa, S. (1974) *J. Biol. Chem.* **249**, 1889–1896
19. Foster, D. W., and Ginsburg, V. (1961) *Biochim. Biophys. Acta* **54**, 378–381
20. Kornfeld, R. H., and Ginsburg, V. (1966) *Biochim. Biophys. Acta* **117**, 79–87
21. Greenwood, F. C., Hunter, W. M., and Glover, J. S. (1963) *Biochem. J.* **89**, 114–123
22. Briles, E. B., Gregory, W., Fletcher, P., and Kornfeld, S. (1979) *J. Cell Biol.* **81**, 528–537
23. Ginsburg, V. (1961) *J. Biol. Chem.* **236**, 2389–2393
24. Glaser, L., and Zarkowsky, H. (1971) in *The Enzymes* (Boyer, P. D., ed) Vol. 5, pp. 465–480, Academic Press, New York
25. Gabriel, O. (1973) *Adv. Chem. Ser.* **117**, 387–410
26. Yurchenco, P. D., and Atkinson, P. H. (1977) *Biochemistry* **16**, 944–953
27. Borberg, H., Yesner, I., Gesner, B., and Silber, R. (1968) *Blood* **31**, 747–757
28. Kaufman, R. L., and Ginsburg, V. (1968) *Exp. Cell Res.* **50**, 127–132
29. Cox, R. P., and Gesner, B. M. (1965) *Proc. Natl. Acad. Sci. U. S. A.* **54**, 1571–1579
30. Jabbal, I., and Schachter, H. (1971) *J. Biol. Chem.* **246**, 5154–5161
31. Gaugler, R. W., and Gabriel, O. (1973) *J. Biol. Chem.* **248**, 6041–6049
32. Beyer, T. A., Rearick, J. I., Paulson, J. C., Prieels, J.-P., Sadler, J. E., and Hill, R. L. (1979) *J. Biol. Chem.* **254**, 12531–12541
33. Allen, A. K., Desai, N. N., and Neuberger, A. (1976) *Biochem. J.* **155**, 127–135
34. Watkins, W. M. (1972) in *Glycoproteins* (Gottschalk, A., ed) Part B, pp. 830–891, Elsevier, New York
35. Remold, H. G. (1973) *J. Exp. Med.* **138**, 1065–1076
36. Poste, G., Allen, H., and Matta, K. L. (1979) *Cell. Immunol.* **44**, 89–98
37. Higgins, T. J., Liu, D. Y., Remold, H., and David, J. R. (1980) *Biochem. Biophys. Res. Commun.* **93**, 1259–1265
38. Prieels, J.-P., Pizzo, S. V., Glasgow, L. R., Paulson, J. C., and Hill, R. L. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 2215–2219
39. Stahl, P., Schlesinger, P., Shepard, V., Tietze, C., and Townsend, R. (1980) *Fed. Proc.* **39**, 1968
40. Rosati, R., and DeSantis, R. (1980) *Nature* **283**, 762–764
41. Cheng, C. C., and Bennett, D. (1980) *Cell* **19**, 537–543
42. Gesner, B. M., and Ginsburg, V. (1964) *Proc. Natl. Acad. Sci. U. S. A.* **52**, 750–755